♣ Evaluation of Some Approaches to Liquified Tallow: Stereochemical Consequences of Interesterification

Several approaches to converting tallow to a liquid state at ambient temperature to facilitate incorporation into poultry feed were examined. Acetone fractionation can be used in a single step to produce a flowable powder and an oil (no additional semi-solid fraction). Partial hydrolyses and interestifications mediated by lipases of *Candida rugosa, Rhizopus delemar* and porcine pancreas did not lead to liquefaction. Interesterification (trioctanoin + glycerol, 1,2-acetonide) in the presence of *C. rugosa* lipase shows a small but significant sterobias indicating that there are stereochemical consequences in such processes that deserve further investigation.

There is a renewed interest in altering the physical properties of tallow so that it might be more satisfactorily used, as an additive for poultry feed (Private communications with D. Gilcrest, president, Fats and Proteins Foundation, Inc., Des Plaines, Illinois). Feed grade animal fat, or yellow grease, is collected from restaurants, as residue from food preparation. Its constitution is therefore both animal and vegetable in origin and is quite variable. It will separate into liquid and semi-solid on standing in much the manner of various grades of tallow. The current process of blending yellow grease to produce a feed would be better served if the feedstock to the mills was entirely liquid; the semi-solid material reportedly hampers the milling process. Chemical fractionation, or alteration, of the yellow grease is one of several alternatives that could accomplish this goal. Because fancy bleached tallow has the highest titer, we felt that any procedure that liquefied the tallow would succeed with tallow-containing mixtures. We report here our results using the several possible chemical approaches to liquefaction with fancy bleached tallow.

MATERIALS

Acetone and acetonitrile were Mallinckrodt Nanograde. Tallow was a gift of Chemol Inc., Greenboro, North Carolina; corn oil (Mazola) and sunflower oil (Wesson) were local purchases; and soy oil was obtained from Central Soya, Decatur, Indiana. Tributyrin (Eastman, Rochester, New York), and tris-heptafluorocamphoratoeuropium, or Eu(hfc)₃ (Aldrich, Milwaukee, Wisconsin), were used directly as obtained. Trioctanoin (Eastman, Rochester, New York) was treated with a small amount of octanoyl chloride and pyridine to esterify the dioctanoins present and precipitated several times from cold alcohol. The acetonide of glycerine was synthesized from acetone and glycerol; product bp 51-53 C/0.1 mm. The lipases used were from *Candida rugosa* (Enzyme Development Corp., New York, New York), *Rhizopus delemar* (Seikagaku Kogyo Co. Ltd., Tokyo, Japan), and porcine pancreas (Sigma Chemical Co., St. Louis, Missouri). High performance liquid chromatography was performed with a Perkin Elmer Series 2 pump, a Waters Associates differential refractometer and an ODS (reverse phase) column (4.6 mm × 25 cm). Gas liquid chromatography of

TABLE 1

Acetone Fractionation of Tallow

T^a	% Solid	Melting range	T^b	CP
25	4.4	54-56	>25	23.5
20	8.0	50-53	25	16
15	10.7	49-53	21	13
4	27.1	35-47	19	9

^{a}T, T C for crystallization.
^{b}Minimum T C at which recovered oil remained liquid for 48 hr.
^{c}P, cloud point.

fatty acid methyl esters was accomplished using Shimadzu-Mini GC-2 with a SPB-1 column (0.25 mm ID × 30 m) operating at 240 C. Titrations for free fatty acid were obtained with a Radiometer pH unit comprised of an ABU 80 Autoburette module. Proton NMR spectra were obtained in $CDCl_3$ solution at 400 MHz with a JEOL GX400 spectrometer.

Solvent fractionation. A detailed study of acetone fractionation of edible beef tallow was accomplished several years ago (1). Its purpose was to produce a fraction with physical properties akin to those of cocoa butter. The procedure provided two solid fractions comprising 14% of the original batch of tallow and two liquid fractions of 66%. The remaining 20% was a semi-solid similar to cocoa butter. From this was developed a continuous process to obtain a confectionery fat from tallow (2) that eventually was patented (3). This fractionation procedure for cocoa butter suggested that a one-step process to separate tallow into a flowable powder and a liquid (no semi-solid fraction) was possible.

The results of such a one-step crystallization are summarized in Table 1. Chemol tallow was dissolved in Nanograde acetone (50 g/500 ml) and the solutions allowed to stand for 24 hr at a specific temperature. The precipitate was filtered and washed with 50 ml of acetone chilled to 0 C. The weights of recovered solids and oils were recorded, and the lowest temperatures at which the recovered oils could be kept for 48 hr as liquids were determined. The proportion of solid recovered increases with the lowered temperature for cyrstallization as expected, and the recovered liquid fraction survives as a liquid to a lower temperature. Because the triglyceride content in the fractionations originally reported (1) was quite detailed there seemed no need to repeat this. However, triglyceride analysis by HPLC (4) has become routine in the interim, and we did examine the recovered oils to qualitatively determine the triglycerides that are selectively lost to crystallization (examination of the solid fractions directly by HPLC runs the risk of precipitating the solid on the HPLC column (Fig. 1). The triglycerides that are deposited at higher crystallization temperatures are primarily PPS (PSP) and PSS (SPS). At lower crystallization temperatures one obtains additionally PPP, POS and SOS. Consistent with these observations were the analyses of fatty acid methyl esters that were obtained by transesterification with sodium methoxide (5) (Table 2). Solids deposited at higher temperatures are richer in stearic and palmitic relative to oleic, but as precipitation temperature is lowered a greater proportion of oleic acid
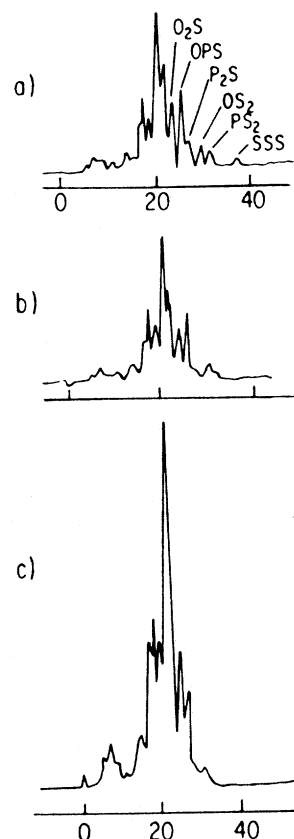

FIG. 1. Chemol tallow fractionation. ODS column (1/4" × 25 cm), CH_3CN at 0.3 ml/min; a, tallow itself; b, recovered oil after crystallization at 25 C; c, recovered oil after crystallization at 4 C. Time is in minutes.

is present in the solid triglycerides.

Although a process that separates tallow into a flowable solid and a liquid with high recovery certainly seems possible, the costs associated with introducing equipment for a large scale fractionation and solvent recovery may be prohibitive. Moreover, additional concerns must be addressed to meet the safety requirements for handling flammable solvent.

Enzymatic partial hydrolysis. Enzymatic hydrolysis (lipolysis) of fats and oils has been established as an alternative to conventional splitting that is less energy intensive, shows selectivity, and is much milder than current methodology (6). Although tallow is more resistant to complete hydrolysis (7), it is evident that sufficient hydrolysis can be affected by lipase to

TABLE 2

Fatty Acid Composition of Fractions from Acetone Precipitation

	Oil phase									Solid				
T	14:1	14	16:1	16	17:1	17	18:2	18:1	18	T	14	16	18:1	18
25	2.9	21.7	6.3	52.2	0.3	0.4	2.3	11.0	2.9	25	6.0	59.3	2.2	32.4
20	3.1	24.2	7.3	49.4	0.2	0.2	2.2	11.1	2.4	20	5.9	56.7	7.5	29.9
15	4.4	19.0	7.1	48.5	0.3	0.5	3.6	13.2	3.4	15	8.2	54.8	6.6	30.4
4	4.0	23.7	7.9	45.6	0.7	1.8	2.4	11.9	2.0	4	7.5	55.6	15.0	21.9

determine whether the residual mixture of glycerides at any point is a liquid at ca. 25 C. The positionally nonselective lipase of *Candida rugosa* and the 1,3-selective lipase of *Rhizopus delemar* were used to affect a partial hydrolysis of tallow. In each instance 3.0 g of tallow and sufficient water to cause a limited hydrolysis were exposed to 10 mg of commercial *C. rugosa* lipase (867 U/mg) (8) or 3 mg of commercial *R. delemar* lipase (1030 U/mg) for a period of 16-20 hr at 37 C. Activity was determined by hydrolyzing olive oil in pH 7.0 phosphate buffer at 37 C after a brief sonication. U = mmol FFA/mg, hr. The mixtures were titrated for free fatty acid and duplicates were worked up to obtain the glyceride fraction free of the acids. The recovered oils were the products of up to 44% hydrolysis (*C. rugosa*) and 30% (44% of theoretical) (*R. delemar*), and all of these became semisolids at 25 C. The presence of significant amounts of saturated C_{16} and C_{18}, particularly at the 1- and 3-glycerol positions, is thought to be responsible for the high titer of tallow. The available lipases, however, show uniformly good reactivity with oleoyl residues and much less reactivity with precisely the palmitoyl and stearoyl residues that must be removed. Thus, in retrospect, it is not surprising that the oils recovered from partial lipolysis of tallow do not exhibit a lower titer.

Enzymatic interesterification. Simple physical blending of various oils such as soy, and corn oil with tallow in various proportions, fails to produce materials that remain liquid at 25 C. Indeed, yellow grease itself is a physical mixture of tallow with various lower melting triglycerides and similarly deposits the higher melting triglycerides. Recent overviews of lipase-catalyzed interesterifications of oils and fats (9, 10) indicated the potential for exchanging fatty acid residues to produce higher unsaturation and/or shorter chains in the tallow. One approach uses oleic acid as a source of titer-reducing oleoyl units that could be introduced at the 1,3- or 1,2- and 3-positions by suitable choice of lipase. Such a procedure would produce a fatty acid mixture containing oleic acid that would eventually require processing to recover unused oleic acid. In addition, the cost of the acid(s) used for such a process would have to be balanced by the cost of the product; in this case the product projected was poultry feed. In another procedure one can use mono- or diglyceride, or a triglyceride (or oil) in conjunction with a small amount of water to provide the alcohol (glycerol hydroxyl), to mediate acyl exchange. In fact, palm oil has been similarly liquified by replacing 40-50% of its palmitic acid using a lipase prepared from *C. deformans*, though details are not reported (11).

In order to visualize a projected interesterification process for tallow more clearly, equimolar mixtures of trimyristin and triolein were exposed to *C. rugosa* lipase with water sufficient to cause 10% hydrolysis. Reactions were conducted at 40 C. The processes of acyl migration were easily followed by HPLC (Fig. 2) and continued long after maximum hydrolysis had been achieved. In these experiments (150 mg of the lipase to 3.33 mmol of each triglyceride) the final disposition was achieved in about 120 hr.

Tallow was allowed to react with tributyrin in various proportions from 10% to 50% by weight at 40 C

mediated by porcine pancreatic lipase, and using sufficient water (pH 7.0 buffer) to cause 10% hydrolysis. The amount of lipase used was 130 mg ($\sim$ 9500 U) per 2.8 g of tallow, and 100 mg of lipase was added daily until reaction time attained 120 hr. In each reaction at least one-half of the butyryl residues had become incorporated into the tallow triglycerides (HPLC analysis of residual tributyrin), presumably replacing acyl residues at the primary positions. The recovered triglycerides, however, solidified at 25 C directly in all cases. In a similar experiment tallow was allowed to react with equal weights of corn, soy and sunflower oil catalyzed by
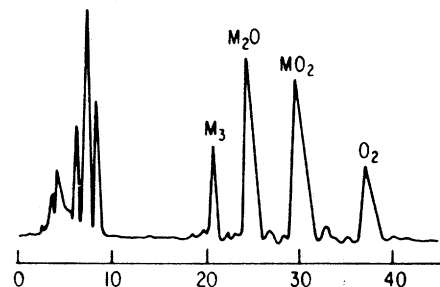

FIG. 2. Interesterification of trimyristin (M_3) and triolein (O_3): *C. rugosa* lipase, 40 C, 120 hr, ODS column (1/4" $\times$ 25 cm), acetone CH$_3$CN (64:36), 1.0 ml/min. Time is in minutes.
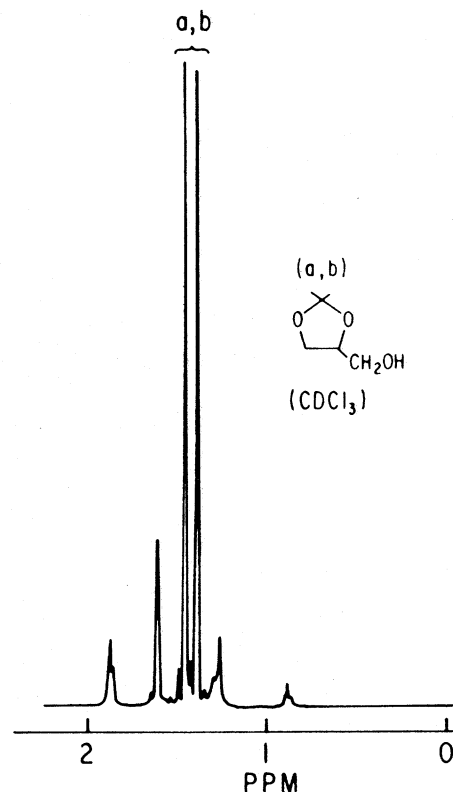

FIG. 3A. ^{1}H NMR spectrum of 1,2-acetonide of glycerol recovered from lipase catalyzed esterification obtained by Fourier transformation of four accumulated scans consisting of 32,768 data points in a 10-KHz spectral window using 0.6 Hz broadening factor. Data were acquired with a 90° pulse (6.3 μs) and a total pulse recycle delay of 3.6 seconds. Horizontal axis is in ppm relative to TMS.

porcine pancreatic lipase. Again the recovered triglyceride mixtures did not remain liquid. In all of these studies enzymatic activity was still present at the conclusion of an experiment; this was demonstrated by adding water and observing further hydrolysis.

Though interestification of tallow occurs, the extent of replacement of selected acyl groups in order to achieve a particular goal, in this case a liquified form of the tallow, appears to be considerable. Simple redistribution using an available liquid fat (vegetable oil) may provide other useful blends and may be a means to achieve a semi-solidification of the oil. However, as a method to liquify the tallow, interesterification may not be useful. Replacement of saturated C_{16} and C_{18} with shorter chains to achieve this goal also has not been demonstrated. More importantly, the natural enzymes do not react readily with those particular acyl groups that require displacement. In addition, the esterification process intrinsically obligates the enzyme to react with aliphatic acids, or requires the acylated enzyme to react with a diglyceride. These are not natural substrates and nucleophilic species, respectively, and large amounts of the lipases appear to be required to carry out relatively slow reactions.

Interesterification and stereochemistry. Porcine pancreatic lipase reportedly reacts without a stereobias with triglycerides (12), though recent literature describes evaluations of kinetic resolutions of esters by several lipases (13–15). The esters used are structurally dissimilar from triglycerides, and the closest structure exposed to lipolysis (porcine pancreatic lipase) was the butyrate ester of glycerol, 1,2-acetonide (14). Lipolysis of this ester was taken to 60% conversion; the recovered butyrate was roughly a 69:31 mixture of enantiomers. This indicates approximately a 2:1 rate ratio (16). The stereoselective esterification of alcohols using tributyrin as a solvent also has been described (17) and indicates the considerable stereobias of a lipase (*C. rugosa* lipase in this case) in the step whereby acylated enzyme reacts with nucleophile. In view of these documentations and the apparent slowness and incompletion of esterifications (18,19), we were prompted to examine the esterification of the 1,2-acetonide of glycerol arbitrarily using *C. rugosa* lipase and one equivalent of trioctanoin. The choices of reagents were as follows: the acetonide is a readily available analog of a diglyceride that would not itself react enzymatically though its structural dissimilarity to a true diglyceride is apparent. Also, results might be compared to those reported for the hydrolysis of the corresponding butyrate (14). The trioctanoin is liquid at room temperature, as is the glycerol derivative, affording a stirrable single phase. The *C. rugosa* lipase was deposited onto controlled pore glass (375 Å); this formulation was granular and, unlike a celite support, remained easily stirred. The reaction was followed by HPLC and terminated at 81.6% conversion. Unreacted glycerol 1,2-acetonide was recovered by distillation and determined to contain an enantiomeric excess of 15% (Figs. 3A,3B) using the NMR chiral shift agent, Eu(hfc)₃. Use of this reagent to determine the configurational bias in such compounds has been reported (15), though details are yet to be published. Our data indicate that the rate ratio for octanoylation of the glycerol derivative is less than the 2:1 reported for debutyrylation (ratio of
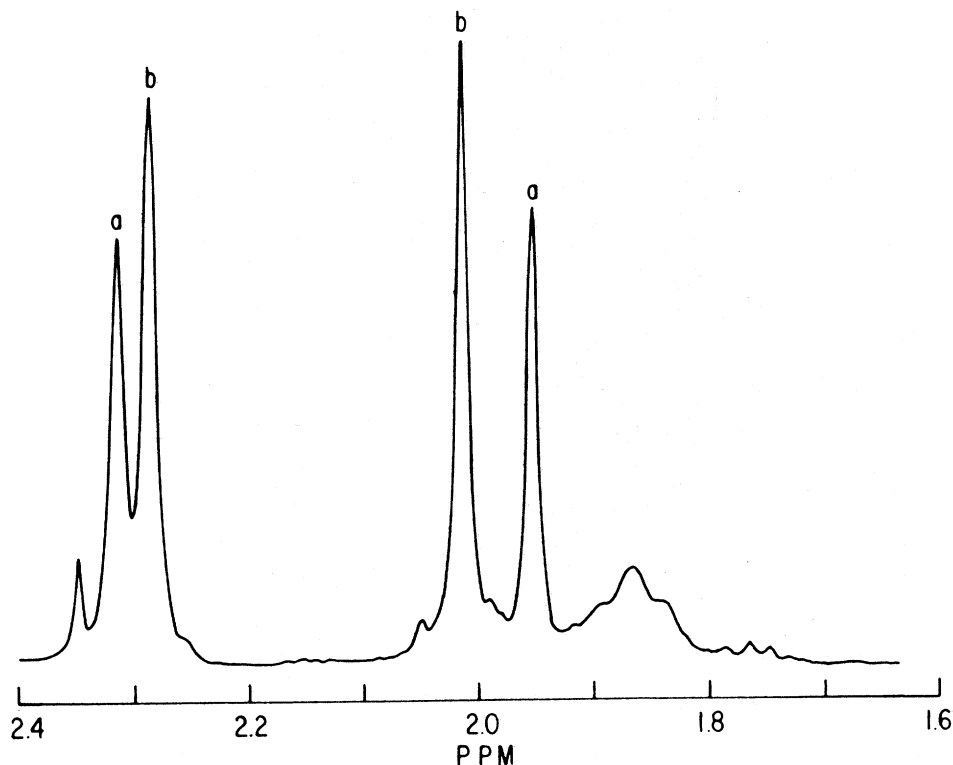

FIG. 3B. Samples described in 3A to which 0.3 equivalents of a chiral shift reagent, Eu(hgc)₃, had been added showing differentiation (and unequal areas) of the gem dimethyls of the enantiomers.

rates $\sim$ 1.19). This could be ascribed to the fact of the change in lipase or the acid chain length. Because of the importance of interesterification chemistry to possible commercial processes, a more detailed investigation of the stereochemical consequences of this process seems warranted and is under way. In addition, we have made use of lypase stereobias in esterification and interesterification to synthesize insect sex attractants (20).

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